Plasmid profiles compared with serotyping and pyocin typing for epidemiological surveillance of *Pseudomonas aeruginosa*

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Summary. The plasmid profiles of 112 clinical isolates of *Pseudomonas aeruginosa* were determined by two reproducible, rapid, plasmid screening methods. Plasmid DNA was present in 15% of isolates examined. Plasmids varied in size from 1.2×10^6 to 60.2×10^6 mol. wt. The dominant serotypes encountered were O:11 and O:4, which comprised 38% and 12% of strains, respectively. Four pyocin types (1, 10, 3 and 5) dominated (respective frequencies: 56, 15, 12 and 6%). Reproducibility of pyocin typing was distinctly inferior to both plasmid profiling and serotyping. Strains of identical serotypes could be further differentiated by dissimilar plasmid profiles. Serologically untypable or polyagglutinable strains were successfully characterised by plasmid profile patterns. Thus, plasmid profiling was shown to be a useful adjunct to serotyping for the epidemiological typing of *P. aeruginosa*.

Introduction

The importance of *Pseudomonas aeruginosa* as an opportunist pathogen causing nosocomial infections is well established (Bodey *et al.*, 1983). Typing systems have been used extensively to trace the routes of infections within hospitals. Conventional systems like serotyping, phage typing and pyocin typing have merits and limitations (Bergan, 1975). Lack of consistent results and the elaborate work required to maintain typing phage preparations have made phage typing only moderately useful for *P. aeruginosa* (Bergan, 1978); poor reproducibility has been of some, although lesser, concern in the case of pyocin typing (Fyfe *et al.*, 1984). Serotyping gives better reproducibility (Lányi and Bergan, 1978) but is unsatisfactory for mucoid strains of *P. aeruginosa* (Williams and Govan, 1973). In contrast, diverse but readily classifiable plasmid profile patterns have been described for some bacteria, e.g., within *Salmonella* spp. (Bezanson *et al.*, 1983; Holmberg *et al.*, 1984), gram-negative bacilli (Schaberg *et al.*, 1981), *Campylobacter* spp. (Bradbury *et al.*, 1983) and *Legionella* spp. (Maher *et al.*, 1983).

Accordingly, this study was designed to assess the potential usefulness of plasmid profiles for epidemiological typing of *P. aeruginosa* and to

compare this method with serotyping, pyocin typing and antibiogram typing.

Materials and methods

Bacterial strains

One hundred and twelve isolates of *P. aeruginosa* collected from seven hospitals in Singapore were studied. Two of the hospitals contributed most of the strains (67 isolates from Singapore General Hospital, 34 from Tan Tock Seng Hospital, the remaining five hospitals contributing 11 strains). Specimen sources were wounds, 65; urines, 20; respiratory tract, 14; remainder from blood, abscess, pus, peritoneal fluid, catheter tip, ear and urethra. Strains were maintained with bi-weekly subcultures on slopes of Nutrient Agar (Scotts Laboratories, USA).

Conventional typing schemes

All strains were serotyped according to the International Antigenic Typing Scheme (Liu *et al.*, 1983) with commercial sera produced by Difco Laboratories (USA). Autoclaved cells from C-N Agar (Oxoid) were tested by slide agglutination. Pyocin typing by cross-streaking was performed as described by Gillies and Govan (1966). J. R. W. Govan kindly provided indicator strains and four standard producer strains (PP 430, F1, Creek 7390, Sands 3398) which were included as reference controls when testing clinical isolates.

Antimicrobial susceptibility was determined by the Bauer-Kirby method (Bauer *et al.*, 1966).

Pyocin typing was performed at least three times and serotyping at least twice on each isolate.

Isolation of plasmid DNA

Cells were grown in 10 ml of Trypticase Soy Broth (Difco) overnight at 37°C. The plasmids were extracted by two methods, that of Portnoy *et al.* (1981) and the alkaline dodecyl sulphate lysis method of Kado and Liu (1981).

Agarose gel electrophoresis

Samples (30–40 µl) containing plasmid DNA were mixed with 10 µl of bromocresol purple 0.25% in 50% glycerol–0.05 M Tris-acetate (pH 7.9) on a sheet of Parafilm "M". DNA samples (40–50 µl) were electrophoresed through 0.7% agarose on a horizontal slab gel system in a Tris-borate buffer. The gel was run at 20V for 16 h at room temperature. Low voltage electrophoresis was used for screening to enable identification of small plasmids of mol. wt < 10 × 10⁶. Gels were stained with 100 µl of a solution of ethidium bromide 10 mg/ml for 1 h, then placed on a short-wavelength UV light box (Model C-61, Ultraviolet Products, Inc., San Gabriel, CA, USA) and photographed with a Polaroid CU-5 camera with UV filters and type 665 Polaroid film.

Plasmid reference markers

Escherichia coli strains carrying plasmids of known mol. wts were obtained from Dr Esther Lederberg, Plasmid Reference Centre, Stanford University School of Medicine. They were *E. coli* strains V517 (containing plasmids with mol. wts (10⁶) in the range 1.4–35.8), RP4 (plasmid mol. wt 36 × 10⁶) and TP116 (plasmid mol. wt 143 × 10⁶). Mol. wts of the crude plasmid preparations were estimated by comparing their electrophoretic mobilities with those of the reference plasmids on horizontal agarose gels.

Results

Serotyping

The commonest serotypes were O:11, 4, 5, 3 and 6, with respective frequencies of 37.5, 10.7, 8.0, 8.0 and 7.1% (table I). The predominant serotype O:11 was subdivided primarily into two major pyocin types (PT), PT1 and PT10, but 19% were either unclassified (UC) or untypable (UT). The same pyocin types dominated also amongst the O:4 strains (eight strains of PT1, two of PT10 and two UT). Strains of the serotypes O:3, 5 and 6 belonged mostly to other pyocin types (serotype O:3 included five strains of PT5 and four of either UC or UT;

Table I. Distribution of *P. aeruginosa* serotypes

Serotype	Number (%) of strains of given serotype
1	1 (0.9)
2	2 (1.8)
3	9 (8.0)
4	12 (10.7)
5	9 (8.0)
6	8 (7.1)
7	2 (1.8)
8	2 (1.8)
9	1 (0.9)
10	6 (5.4)
11	42 (37.5)
12	5 (4.5)
13	0 (0)
14	0 (0)
15	0 (0)
16	1 (0.9)
17	0 (0)
NA	10 (8.9)
PA	2 (1.8)

NA = non-agglutinable; PA = polyagglutinable.

serotype O:5 included six strains of PT1, two of PT3, one of PT6; serotype O:6 included seven strains of PT3 and one of PT27).

Pyocin typing

The commonest pyocin types were PT1, PT10, PT3 and PT5, with respective frequencies of 40.2, 22.3, 13.4 and 4.5% (table II). Of the 112 clinical isolates typed, 5.4% belonged reproducibly to type patterns not included in the type chart described by Govan (1978). Because of variable type patterns upon repeated typing, 10.7% of the strains were assigned as UC (unclassified) types. This situation occurred mostly with weak pyocin producers and particularly involving indicator strain no. 6, which showed varied responses in such cases and was responsible for fluctuation between PT1 and PT10 and changes between PT3, PT22 and PT27. Subtype results were even less reproducible. Only 69% of the isolates fell into the same pyocin subtype on at least three repeated typings.

Plasmid profiling and traditional epidemiological markers

Plasmid extraction by both procedures gave consistent results on repeated extractions and electrophoreses. Plasmids were detected in the same 17 strains (15% of total isolates) by both methods. A total of 11 different plasmid profiles (PP) were observed (table III). Three plasmid

profiles occurred in two or more strains. The first, PPI, consisted of plasmids of mol. wt (10^6) 1.2 and 1.5; the second, PPII, of a plasmid of mol. wt 18×10^6 , and the third, PPIII, of a plasmid of mol. wt 31.6×10^6 . PPI was observed in five strains (nos. 40, 81, 88, 104 and 112) as shown in fig. 1 (lanes A, H, J, L and M respectively); all these came from one hospital and belonged to the same (O:11) serotype. Although their pyocin types differed, derivation from the same original source is possible in view of the observed fluctuations in pyocin types and particularly the inconsistency of the inhibition of indicator strain no. 6. PPII was observed in two strains (nos. 84 and 102; fig. 2, lanes H and K) which were epidemiologically unrelated, being derived from two hospitals, and of different serotypes. The third profile, PPIII, was characterised by a 31.6×10^6 mol. wt plasmid in two strains (nos. 8 and 76; fig. 2, lanes A and G) but they were epidemiologically unrelated, being of different serotypes and pyocin types, and from differing hospital sources.

Different plasmid profiles were found in isolates belonging to the same serotype (strains nos. 29, 40, 48, 76, 84, 86 and 91; table III) and, conversely, identical plasmid profiles were found in two strains belonging to two different serotypes (strains nos. 84 and 102). Three strains of serotype O:11 and PT:UT (strains nos. 84, 88 and 91) could be

Table II. Distribution of *P. aeruginosa* pyocin types

Pyocin type*	Number (%) of strains of given pyocin type
1	45 (40.2)
10	25 (22.3)
3	15 (13.4)
5	5 (4.5)
Others†	4 (3.6)
UC	12 (10.7)
UT	6 (5.4)

UC = unclassified types. UT = untypable.

* Pyocin types according to the scheme of Govan (1978).

† Minor pyocin types.

differentiated by their plasmid profiles. Plasmid profiles also differentiated three strains of serotype O:11 and PT:1/h (strains nos. 48, 76 and 112).

When the plasmid-containing strains were classified on the basis of their antibiograms, three patterns were evident. Pattern no. 1 was defined as susceptibility to all agents tested; pattern 2 showed resistance only to streptomycin; and pattern 3 was characterised by multiple resistances. Despite similarities in antibiogram and serotype, the two strains of pattern 1 (strains nos. 8 and 10, table III) were of different pyocin types and plasmid profiles. Of the eight strains of antibiogram pattern 2 the

Fig. 1. Plasmid profiles of clinical isolates of *P. aeruginosa*: profile I in strains nos. 40 (lane A), 81 (lane H), 88 (lane J), 104 (lane L) and 112 (lane M). Mol. wt standard plasmids from *E. coli* strains V517 (lane D; mol. wts (10^6) 1.4-4.8) and RP4 (lane E; mol. wt 34×10^6). Arrow indicates chromosomal band.

Fig. 2. Plasmid profiles of clinical isolates of *P. aeruginosa*: profile II in strains nos. 84 (lane H) and 102 (lane K); profile III in strains nos. 8 (lane A) and 76 (lane G). Mol. wt standard plasmids from *E. coli* strain V517 (lane L; mol. wts (10^6) 1.4–4.8) and plasmid TP116 (lane M; mol. wt 143×10^6).

Table III. Epidemiological markers of clinical isolates of plasmid-containing *P. aeruginosa*

Strain no.	Serotype	Pyocin type	Antibiogram							Mol. wt of plasmids (10^6)	Plasmid profile
			Sm	Gm	Si	Tb	Cb	Ne	Ti		
40	O11	10/a	R	S	S	S	S	S	S	1.2, 1.5	I
81	O11	10/a	NT	NT	NT	NT	NT	NT	NT	1.2, 1.5	
88	O11	UT	R	R	NT	R	R	R	R	1.2, 1.5	
104	O11	10/h	R	R	NT	R	R	R	S	1.2, 1.5	
112	O11	1/h	R	R	NT	R	R	R	R	1.2, 1.5	
84	O11	UT	R	S	S	S	S	S	S	18	II
102	O3	UT	R	S	S	S	S	S	S	18	
8	O6	27/e	S	S	S	S	S	S	S	31.6	III
76	O11	1/h	R	S	S	S	S	S	S	31.6	
91	O11	UT	R	R	NT	R	R	R	S	1.2, 1.5, 19.5	IV
48	O11	1/h	R	R	R	S	R	NT	NT	1.2, 1.5, 60.2	V
86	O11	1/UT	NT	NT	NT	NT	NT	NT	NT	1.2, 1.3, 1.5, 41.7	VI
72	UT	1/d	R	S	S	S	S	S	S	1.2, 41.7	VII
29	O11	10/UT	R	S	S	S	S	S	S	4.7	VIII
10	O6	3/e	S	S	S	S	S	S	S	15.8	IX
69	PA	UT	R	S	S	S	S	S	S	6.8	X
20	UT	3/1	R	S	S	S	S	S	S	30.9	XI

Sm=Streptomycin, Gm=gentamicin, Si=sisomicin, Tb=tobramycin, Cb=carbenicillin, Ne=netilmicin, Ti=ticarcillin. R=resistant, S=susceptible, UT=untypable, PA=polyagglutinable, NT=not tested.

same plasmid profile (a single plasmid of mol. wt 18×10^6) was found in only two strains. Two small plasmids of mol. wts (10^6) 1.2 and 1.5 were detected in several multiresistant isolates with different antibiograms. These two plasmids were also found in a strain of pattern no. 2.

Discussion

Control of nosocomial infections necessitates surveillance programmes designed to identify epidemic strains. The use of plasmid profiles as an epidemiological tool has gained impetus during the past 3–4 years. Accordingly, this study was initiated to assess the usefulness of such patterns in the epidemiological typing of Singapore hospital isolates of *P. aeruginosa*.

The distribution of serotypes, with 38% of isolates belonging to O:11 and 11% to O:4, is unusual compared with reports from elsewhere. For instance, in twelve other countries, the frequencies of O:11 strains were in the range 0–16% with a mode frequency of 7% (Lányi and Bergan, 1978); serotype O:4 only contributed 0–4% with a mode of 1%. The most common serotype elsewhere is usually O:6 with frequencies of 4–35% (mode 23%), compared with 7% in Singapore. In some places, the cross-reacting O:2 and O:5 serotypes are important with a combined frequency of up to 42% (mode: 13.5%), compared with 11% in Singapore.

The distribution of pyocin types, on the other hand, is similar to the predominance of PT1, PT3, PT5 and PT10 reported from other centres (Gillies and Govan, 1966; Tagg and Mushin, 1971).

Reliance on the antibiogram as a sole epidemiological marker requires caution. It is most useful if an "epidemic" strain carries a specific resistance marker that readily differentiates it from other strains (Archer and Mayhall, 1983). Its importance is diminished in situations where transfers of antibiotic-resistance plasmids occur. The dissemination of a single "epidemic" R-plasmid was found to cause strains of a single genus and even several genera to become multiresistant and display a common antibiogram (Tompkins *et al.*, 1980). Disparity can also arise in the application of antibiogram determination or in their interpretation (Christensen *et al.*, 1983). In view of the high frequency of multiresistant *P. aeruginosa* strains and the well established variability of antibiogram pattern (Acar, 1980), we would place low priority on the use of antibiograms for nosocomial infection tracing purposes.

Plasmid DNA was detected in only 15% of the 112 clinical isolates. This low proportion limited

the usefulness of plasmid profiling as a sole tool for tracing of nosocomial routes of bacterial transmission. Only 20% of the multiresistant strains from this study carried plasmids. Consequently, the resistance genes are likely to be chromosomally located.

The reproducibility of the plasmid profiles is of importance. Profiles can change, the likelihood of this depending on the stability of the plasmids concerned. Highly unstable plasmids would preclude the use of plasmid profiling as an epidemiological marker. However, recent studies with *S. muenchen* (Taylor *et al.*, 1982) showed that plasmid profiles were stable over a period of 3 months. During the year-long period of this investigation, no loss of plasmids was observed. However, changes in plasmid profiles (multiple forms and concatemers) were occasionally observed. These could be attributed to variations in the conditions of extraction of plasmid DNA and the conditions of electrophoresis.

Plasmid profiles offered a new alternative typing method. Among the eleven PPs observed, only three occurred in more than one strain. Of the eleven plasmid-carrying strains of serotype O:11, five (strains nos. 40, 81, 88, 104 and 112) were epidemiologically related. Two strains (nos. 88 and 104) isolated from the same patient on successive occasions were serologically identical and had identical plasmid profile but belonged to two different pyocin types. In this case, the different pyocin types would have to be interpreted as being within the margin of biological variation due to the erratic responses of one pyocin indicator strain.

The absence of plasmids from the majority (85%) of the strains limited the possibility of demonstrating any correlation between specific serotypes or pyocin types on the one hand and plasmid profiles on the other. Any correlation seems loose. This contrasts with the situation in *C. jejuni* and *C. coli*, in which serospecific plasmid profiles have been reported (Bradbury *et al.*, 1983).

The usefulness of plasmid profiling would be increased if higher frequencies of plasmid-carrying strains should arise. The technique, though, showed considerable potential for subdivision within the single serotype O:11 which dominated in Singapore. Reproducibility was considerably higher than that of pyocin typing. Serotyping successfully distinguished considerably more of the present strains and must maintain its priority as typing method of first choice, at least with plasmid carriage rates as low as in Singapore.

In serological "H" typing schemes, strains belonging to the same O group can be further

subdivided. However, such subdivision entails the preparation of specific H antisera (Pitt, 1981). Similar subtyping of dominant serotypes was achieved in this study by plasmid profiling. Since plasmid profiling and serotyping complement each other, the two methods could be used together. Plasmid profiling is easy to perform and does not

require the elaborate reference system of classical typing methods. Thus, plasmid profiling could improve the accuracy of tracing of nosocomial strains of *P. aeruginosa*.

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